THE INGRESS OF *Pseudomonas alboprecipitans* ROSEN INTO SWEET CORN (*Zea mays saccharata* (STURTEVANT) BAILEY) IN RELATION TO STOMATAL APERTURE AND INFECTION COURT

by

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1979

ACKNOWLEDGEMENTS

I wish to express gratitude to Drs. R. E. Stall and J. O. Strandberg, co-chairmen of my supervisory committee, for their guidance and friendship during the course of this study. I also wish to extend thanks to Dr. R. D. Berger for his suggestions in the analysis of the data. Thanks are also extended to Drs. T. E. Humphreys and D. J. Mitchell for their suggestions and guidance in the development of my program.

Gratitude is expressed to Dr. D. A. Samuelson for his instruction in the use of the scanning electron microscope. I also thank Miss Jean Harris for her assistance in collecting data.

Finally, sincere gratitude is expressed to Patrice, my wife, for her patience and love during the course of my studies, and a special thank you for her efforts in preparing the manuscript in its final form.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
ABSTRACT	iv
INTRODUCTION	1
MATERIALS AND METHODS	10
Identification of the infection court	10
Analysis of potential control strategies	19
RESULTS	23
Evidence for the identification of the infection court	23
Analysis of potential control strategies	56
DISCUSSION	67
LITERATURE CITED	76
BIOGRAPHICAL SKETCH	82

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December 1979

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Events in the infection of sweet corn by *Pseudomonas alboprecipitans* Rosen were studied to assist in the development of control procedures for bacterial leaf blight and stalk rot of corn (BLBSRC). Examination of the infection process of *P. alboprecipitans* provided evidence that leaf areas in or not far removed from the corn-plant whorl were the most favorable sites for infection. The whorl was a favorable environment for bacteria; dew and guttation water collected from the whorl contained nutrients that supported the growth and replication of the BLBSRC pathogen.

The movement of a red-pigmented marker bacterium, *Serratia marcescens* Bizio, was monitored in corn plants over time. Movement of *S. marcescens* confirmed that when applied in the whorl, bacteria were capable of upward movement in leaf tissue as far

as 12.0 cm. A more in-depth analysis of ingress through stripping film autoradiography was done with *P. alboprecipitans* that had been labeled with glucose-^{14}C. The labeled bacteria were aggregated around stomatal areas in whorl tissue. Furthermore, based upon additional evidence obtained with a scanning electron microscope (SEM) it was concluded that stomata were portals of ingress for *P. alboprecipitans*. Bacteria were observed in stomata and sub-stomatal chambers when corn-plant whorls were exposed to 10^9 cells/ml for at least 320 min.

Inoculations of the total plant with *P. alboprecipitans* resulted in the formation of lesions only in leaves emerging from or in the whorl at time of inoculation. Furthermore, a local application of the bactericide phenylmercuric acetate (PMA) in the whorl protected corn plants from the bacteria. Treatment with PMA not only reduced the amount of leaf damage due to *P. alboprecipitans* but also altered the distribution of lesion area. However, because of the short duration of protection (24-48 hr) by PMA, it was concluded that chemical protection of susceptible tissue in the whorl was inefficient for a commercial operation.

Knowledge of the events in the infection of corn was of limited value in the unsuccessful attempts to devise control procedures alternative to chemical control. Prospective bio-control agents were evaluated in planta and in mixed cultures with a streptomycin-resistant mutant (PA 73-31R) of *P. alboprecipitans*. It was concluded that none of the organisms tested would serve as a microbial antagonist to prevent or reduce BLBSRC.

Finally, the manipulation of stomata with abscisic acid (ABA) failed to protect plants from infection. Although mean stomatal apertures of plants treated with ABA were reduced from 4.2 to 0.4 μm, a range of openings occurred up to 1.6 μm. It was concluded that partially opened stomata were sufficiently large portals of ingress for rod-shaped *P. alboprecipitans* (1.5 x 1.0 μm).

INTRODUCTION

Although man has made significant advances in agriculture, he has also created new problems in the name of progress. For example, intensive cultivation and monocultures can provide favorable conditions for pests to multiply. As a result, the use of pesticidal chemicals often has been the only alternative to insure an acceptable yield. However, since the publication of *Silent Spring* (7), there has been a public concern about the impact that pesticides have on the environment. Concern for the environment has stimulated the passage of legislation to regulate the use of pesticides. Knowledge that chemicals cannot provide perfect pest-control combined with the new laws have caused scientists to re-examine pest-control strategies. As a result, man is beginning to adopt a new philosophy of co-existence with pests. In accordance with this idea, the term pest-control has evolved into pest management, an integrated approach to the use of various control methods (35). The purpose of pest management is not the elimination of pesticides, but is a maximization of pest suppression techniques to prevent economic damage to the crop. This includes the effective use of pesticides (41).

Integrated pest management (IPM) is not a magic cure-all which can be applied to all crops for all pest problems. Development of a successful IPM program is dependent upon

accumulation and evaluation of data from basic research. One example of basic information required might be to learn more of the life cycle of certain pests. It is intuitive that blocking or interfering with the completion of the life cycle of a pest would aid in the control of that pest. If economically practical, every opportunity for intervention in the life cycle of a pest should be exploited. Knowledge of key events in the life cycle would assist in the appropriate application of controls as well as in the development of new control strategies. Weak links in the life cycle of many plant pathogens occur between overseasoning and infection of the host. Controls directed against survival of the pathogen, production of inocula, dissemination, and inoculation deal with initial inoculum. Efforts designed to combat prepenetration, penetration, and colonization deal with reducing the rate of disease development.

Control strategies for bacterial plant diseases are often aimed at reducing initial inoculum. This is largely due to the lack of usable bactericides as well as the fact that bacteria often infect juvenile-tissue. Such rapidly growing tissue cannot be continually protected with a pesticide. Unfortunately, controls aimed at initial inocula are sometimes inadequate. Van der Plank (59) proposed that eradication of initial inoculum was least effective against diseases which had a rapid rate of development. If environmental conditions are favorable, low numbers of bacteria which escaped eradication might start an epidemic.

The least exploited points in the life cycle of bacterial plant pathogens have been those events immediately preceding penetration. Although few bactericides are suitable for agricultural crop use, the concept of protection against bacterial infection should be further investigated. On the basis of these concepts, this project was initiated to obtain knowledge of the penetration of *Pseudomonas alboprecipitans* Rosen into sweet corn (*Zea mays saccharata* (Sturtevant) Bailey). It is anticipated that such knowledge will assist us in the development of acceptable control procedures for bacterial leaf blight and stalk rot of corn (BLBSRC). See Gitaitis (18) for a recent literature review of BLBSRC.

Upright grasses such as corn have an efficient system for the catchment and retention of water. Ruinen (48) ascertained that both water from dew and guttation ran down the grass lamina and collected in the whorl of furled leaves and in cisterns formed by the collar and sheath of older, emerged leaves. He noted that excess water overflowed via a tiered system of lamina axils which resulted in water reaching the soil. En route to the soil the water contributed to the leaching of plant substances, some of which served as nutrients for bacteria in the phyllosphere. Water and dissolved nutrients in the whorl and under leaf sheaths provided sites for microbial growth and survival.

Although Ruinen (48) made his observations with saprophytic microorganisms, the concepts involved could be applied easily to plant pathogens. In fact, the microenvironment is a crucial factor in the development of plant disease (66). Haas and

Rotem (22) found that epidemics caused by *Pseudomonas lachrymans* were not limited as much by inoculum levels as they were by factors that controlled ingress of the bacterium. Gitaitis (18) observed that areas of water collection in corn corresponded with areas of symptoms due to *P. alboprecipitans*. Furthermore, foliar lesions of BLBSRC formed only when corn plants were in a vegetative growth stage. The pattern of infection was postulated to be correlated with the disappearance of the whorl as an efficient infection court (18). The whorl area diminished as the plant developed a tassel. Hartman and Kelman (23) likewise proposed that natural infection of corn by an *Erwinia* sp. occurred in the whorl. The authors based their conclusion on the observation that artificial inoculations in the whorl simulated natural field inoculations. However, surfacants were required to approximate natural conditions. Hartman et al. (24) discovered a selective water-soluble inhibitor (DIF) of bacteria that was highest in activity when extracted from whorl areas. Bacterial pathogens of corn were less sensitive to DIF than other plant pathogens. It was concluded that corn pathogens were not susceptible to DIF because they evolved in the presence of the inhibitor. It is of interest that DIF extracts from whorl areas of corn plants were significantly higher in activity than extracts from other corn tissues. If DIF was involved in the evolution of bacterial corn-pathogens, the highest activity in the whorl casts suspicion upon that area as having had a long association with plant pathogenic bacteria.

Several workers cite whorl inoculation as an efficient method to inoculate grasses with bacterial plant pathogens (21, 54, 62). There is also a precedent of the whorl as an infection court for plant diseases of fungal origin. Berger (5) found that weekly fungicidal applications to corn were ineffective against *Helminthosporium turcicum*, causal agent of northern corn leaf blight. Fungicidal applications made 24 hr prior to spore presence were of no value in disease control. The rapid growth of corn plants did not allow fungicides to cover and protect newly emerging tissues in the whorl area. Therefore, the whorl was identified as a primary infection court for the fungus.

Disease development in BLBSRC appeared similar to the above situations. The pattern of blight in natural conditions was as if infection initiated in the whorl. Lesions developed in leaf areas that opposed one another when leaves were furled in the whorl. As a result a horizontal layer of blight occurred throughout the stand. Leaves immediately below the blight layer did not develop symptoms although the leaves were exposed to the inoculum. The implication was that lower leaves were in some condition which decreased their chances to become infected. The most striking difference between upper and lower leaves was that upper leaves were furled in the whorl at the probable time of arrival of inoculum. Because of the importance of the whorl as a possible infection court, an objective of this investigation was to elucidate the function of the corn-plant whorl in relation to BLBSRC. Infection is a crucial event in the life cycle of a plant

pathogen; thus the identification of a specific infection court may have implications for the development of disease control strategies in corn and other grass diseases.

Knowledge of the general site of penetration may be of value for some plant diseases, but specific information is often required to develop effective control procedures. There is no evidence for direct penetration of intact leaf surfaces by bacteria. Consequently, natural openings and wounds are important to bacterial ingress into plants (4, 9, 34, 58). Researchers suggested at an early date that stomata were important portals of entry for bacterial plant pathogens (40, 51). Proof of stomatal ingress usually has been through histological studies of early colonized tissues and not with freshly inoculated material (9, 51). Although Miles et al. (42) displayed excellent scanning electron micrographs of egress of *Xanthomonas pruni* through stomata in lesion areas of peach leaves, evidence of bacteria entering through stomata has apparently never been documented. Therefore, the collection of evidence for bacterial entry of plants through stomata was a major goal of the present study. Techniques such as monitoring the movement of radioisotope labeled bacteria by stripping film autoradiography and scanning electron microscopic examinations of corn tissues were used to collect evidence for stomatal ingress.

If stomatal ingress was the primary mode of entry by *P. alboprecipitans*, then manipulations of stomatal movements could affect disease development. Rich (46) observed that stomatal closing agents protected tobacco from ozone damage and he

speculated that stomatal inhibitors might protect plants from bacterial pathogens. Such an idea may be feasible, as field-scale stomatal manipulations for control of transpiration already have been established (17, 56, 60).

Although the exact mechanisms of stomatal action are not well understood, they deserve a brief review. It is generally accepted that guard cells function as turgor-operated valves (3, 27, 45). Sayre (49) hypothesized that carbon dioxide removal during photosynthesis would raise the pH of the cytoplasm. A rise in pH would activate the hydrolysis of starch to sugar and the subsequent increase in sugar concentration would decrease the water potential, thereby increasing cell turgor. Fischer (16) as well as Humble and Hsiao (27) challenged the starch-sugar hypothesis when they observed an accumulation of potassium ions in guard cells. Osmotic pressure exerted by imported potassium ions could account for the turgor produced in guard cells of open stomata. Although it is generally accepted now that potassium ions serve as the osmotic medium, the exact control mechanism for the process is debatable. Zelitch (69) proposed that glycolate metabolism was the key to stomatal action. Stimulation of noncyclic photophosphorylation via glycolate metabolism would supply ATP for the operation of a potassium ion pump. Raschke (45) argued that stomatal action was based on a hydroactive and hydropassive feedback, both of which were linked with a carbon dioxide feedback system. In Raschke's model, the mesophyll cells would recognize a carbon dioxide depletion due to photosynthesis. Mesophyll cells would send a message

to guard cells as the intercellular carbon dioxide concentration dropped. Guard cells would respond to lower carbon dioxide levels by synthesizing organic acids. Malic acid, the primary organic acid involved, would dissociate into hydrogen and malate ions. A subsequent excretion of hydrogen ions would be accompanied by the influx of potassium ions. Malate would act to balance the potassium influx by ensuring electroneutrality within the guard cells. Therefore, the model is based on the concentration of carbon dioxide in the intercellular spaces so that guard cells would respond indirectly to light (45). However, Mansfield and Meidner (38) reported stomatal opening in some plants was affected by blue light independent of carbon dioxide concentration. Zeiger and Hepler (67) also worked with blue light as they found that onion guard cell protoplasts swelled in response to a blue light stimulus. The authors proposed that light stimulated a membrane transport of electrons that resulted in an influx of potassium ions into the cell.

It is beyond the topic of this study to refute or confirm any hypotheses of stomatal control. It is sufficient to know that compounds such as kinetin (36), phenylmercuric acetate (37, 61), abscisic acid (32, 43), and α-hydroxysulfonates (70) affect stomatal movements. Thus, stomatal inhibitors were used to investigate the effect that stomatal closure had upon ingress of *P. alboprecipitans* into corn leaves.

An alternative control procedure to stomatal manipulation or use of pesticides is bio-control. Saprophytic bacteria which interact with pathogens have been reported for possible

bio-control applications (19, 20, 47, 55, 65). The exact nature of bacterial antagonism is not clear. DeCeara (10) observed that *E. herbicola* grew more rapidly than *E. carotovora* when cultured in vitro. Consequently, faster reproduction and growth of *E. herbicola* altered the growth medium so the availability of nutrients was not only reduced, but also the pH of the medium was modified to below pH 4.5 (10). Therefore, acids produced by *E. herbicola* could have accounted for the inhibitory effects on acid-sensitive plant pathogens such as *E. carotovora* (10) and *E. amylovora* (47). If *E. herbicola* could colonize corn surfaces and alter the microenvironment to be unfavorable for *P. alboprecipitans*, then *E. herbicola* would have potential as a bio-control agent. Consequently, the effect of *E. herbicola* on *P. alboprecipitans* was examined. The precedent of *E. herbicola* as an antagonist to plant pathogens (10, 20) made that organism a likely candidate as a bio-control agent for BLBSRC. In addition, an isolate of *Klebsiella pneumoniae* was also studied as a potential bio-control agent. That organism reproduced and grew at an incredibly fast rate. Therefore *K. pneumoniae* was studied as a possible candidate for bio-control as a competitive inhibitor if not also an antagonistic one. The potential for microbial colonization on the corn plant has been established (48). Therefore if organisms antagonistic to *P. alboprecipitans* could be established in the infection court, they would protect the plant with limited number of applications and be safe for the environment. These criteria would fit into an IPM program if one was developed for sweet corn production in Florida.

MATERIALS AND METHODS

Identification of the infection court

Analysis of the whorl water. Water from corn-plant whorls was tested for either an inhibitory, stimulatory, or neutral effect upon the growth and survival of *Pseudomonas alboprecipitans*. Dew and guttation water that had accumulated in whorls of field-grown sweet corn (cv. Gold Cup) were sampled with a pasteur pipet between 2 and 4 a.m. Plants were in the early whorl stage (fourth leaf visible) at time of sampling. Samples were filter-sterilized with a disposable 0.2 µm millipore filter. The sterile whorl water (SWW) was stored on ice until used (approximately 30 hr). A sample of 0.05 ml of isolate PA 117 (18) of *P. alboprecipitans* was introduced into 20 ml of SWW. The initial population of PA 117 in SWW was determined to be 5×10^3 cells/ml. Three replicates of the cultures were incubated on a rotary shaker for 48 hr at approximately 25 C. After incubation the cultures were serially diluted with a microtiter dilution system (Scientific Products, Waukegen, IL 60085) and the population of PA 117 was determined by the dilution plate method. Estimates of bacterial numbers were based on three replicates.

A portion of SWW was used in experiments in which oxygen uptake by PA 117 was monitored. Consumption of oxygen by PA 117 in whorl water was compared to oxygen consumption in

nutrient broth or 0.2M phosphate buffered-0.85% saline (pH 7.0). Oxygen uptake was measured with a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH 45387). All samples were maintained at a constant temperature of 37 C with a water bath. Rates of respiration of PA 117 in the different solutions were estimated from 3 ml of 10^8 cells/ml, which were monitored for oxygen uptake over 15 min.

The remainder of the SWW was used for qualitative analysis, specifically for the presence of amino acids, sugars, and organic acids. Sterile whorl water was fractionated on cation-anion exchange resin columns. All samples were gravity fed to columns via a 10 cm rubber tube connected to a reservoir flask. Amino acids were eluted first from a cation resin (Dowex 50-X8) column (7.0 mm diameter and 15.0 cm length) with 10 ml of 1N NH_4OH. The amino acid sample was dried and redissolved in 2.0 ml of 10.0% isopropanol. The remainder of the SWW, which had passed through the cation-exchange column, was applied to an anion resin (Dowex 1-X8) column (14.0 mm diameter and 15.0 cm length). Organic acids were eluted from the anion-exchange column with 15.0 ml of 85% formic acid. The sample was concentrated and redissolved in 10% isopropanol to final volume of 2.0 ml. The neutral fraction (SWW which passed through both columns) was concentrated with a rotary vacuum evaporator and tested for the presence of sugars. Quantities of 5-10 µl from all fractions were concentrated in spots on separate pieces of Whatman #3 paper. The amino acid fraction was developed with 0.1% ninhydrin

in acetone. The organic acid fraction was developed with 3 parts of a 1:3 aqueous solution of xylose to 1 part aniline to 100 parts methanol. The sugar fraction was developed with 10 parts 1.0% aniline and 1.0% diphenylamine in acetone to 1 part 85.0% phosphoric acid. Identification of specific compounds was attempted with two-way ascending paper chromatography. All chromatographic procedures were done according to Smith (52).

Movement of Markers. In a preliminary experiment a solution of fluorescent dye (fluoroscein) moved from the whorl into the intercellular spaces of the leaf area above the whorl. An undetermined concentration of aqueous fluoroscein was pipetted into the whorls of corn plants grown in the greenhouse. Plants were subjected to conditions of high relative humidity by intensive watering and enclosure in plastic bags. Plants were exposed to the dye for various undetermined periods of time and then leaves were sampled and viewed with an ultraviolet light. The pattern of dye accumulation was recorded. In order to determine if bacteria as well as dye could move from the whorl into the leaf, a time-course study was conducted. A red-pigmented bacterium (*Serratia marcescens* Bizio) was used to monitor bacterial movements. A 10^8 cells/ml suspension of *S. marcescens* was pipetted into whorls of sweet corn plants (cv. Gold Cup) which were 2-3 weeks of age. Plants were grown in the greenhouse and after inoculation were subjected to conditions of high relative humidity by intensive watering and flooding the greenhouse floor. Emerging leaves were sampled after a 20, 40, 80, 160, and 320 min, as well as after

a 24-hr exposure to the bacteria. Each leaf sampled was aseptically divided into 2.0 cm zones, beginning from the basal end of the leaf. The basal portion of the leaf which was adjacent to the top of the whorl area was designated as the zero reference point. Tissue samples from each leaf-zone were macerated in 0.85% saline from which a loopful was streaked onto nutrient agar. Samples were incubated at 30 C and after 24 hr were examined for the presence of *S. marcescens*.

Autoradiography. Stripping film autoradiography was used as a technique to produce conclusive evidence that *P. alboprecipitans* entered corn leaves through stomata in whorl areas. Radioisotopes have been used previously in determining the distribution of bacteria within plants but never in experiments that have dealt with ingress (53, 63).

Isolate PA 78-5 (isolated from sweet corn at Zellwood, FL) of *P. alboprecipitans* was incubated in nutrient broth which had been supplemented with an unspecific ^{14}C labeled D-glucose (Schwarz/Mann, Orangeburg, NY 10962). Initial radioactivity of the medium was calculated to be 1.0 μC/ml. After incubation for 12 hr, the bacteria were concentrated to a pellet by low speed centrifugation and then were resuspended in sterile water by means of a vortex. The bacteria were washed in this manner with three changes of water. After washing, the bacteria were adjusted photometrically to 10^8 cells/ml (0.3 absorbance at 600 nm in a Spectronic 20 spectrophotometer). The bacterial suspension was concentrated to a pellet by low speed centrifugation and the pellet was resuspended in one tenth the original volume of 0.85% saline, which provided a density of 10^9

bacterial cells/ml. Bacteria (10^9 cells/ml) were pipetted into the whorls of corn plants 2-3 wks of age. Plants were grown in plastic pots in an environmentally controlled greenhouse. All inoculations were done over fiberglass trays lined with absorbent paper. Blocks of tissue (1 cm x 1 cm) from whorl areas were sampled with a disposable razor blade after 6-hr of exposure to the bacteria. Additional samples were taken after symptoms developed (approximately 1 wk). Tissues were fixed in 5.0% glacial acetic acid in absolute alcohol and dehydrated with a standard ethanol-tertiary butyl alcohol dehydration schedule. Dehydrated tissues were embedded in paraffin and sectioned at 10 µm on a rotary microtome. Paraffin sections were affixed with a chrom-alum adhesive to glass slides and expanded on a slide warmer. After section expansion, paraffin was removed with xylene. Fine grain autoradiographic stripping film (AR 10, Eastman Kodak, Rochester, NY 14603) was applied to the radioactive sections. The radioactive isotope in bacteria would expose the film's silver grains, thereby marking the area where the bacteria were located. The film-covered sections were stored in the dark for 8-10 wks, after which the film was developed with Kodak D-19 developer. All procedures were done in a light tight darkroom. After autoradiographs were dry they were examined at 400x and 1000x for developed silver grains which appeared as black dots. All autoradiographic procedures were done over fiberglass trays lined with absorbent paper. Liquid wastes were stored in plastic containers. Absorbent paper and other contaminated solids were stored in plastic bags. All waste was labeled

as radioactive and stored for disposal by the radioactive control officer. See Appleton (2) for autoradiographic procedures and Jensen (28) for histological techniques.

Scanning electron microscopy. Scanning electron microscopy (SEM) was used, in addition to autoradiography, as a technique to obtain evidence of stomatal ingress. Corn plants (cv. Gold Cup) were grown in clay pots under continuous light (6500 lux) and at a constant temperature (28 C) in a model CEL 4-4 controlled environment chamber (Sherer-Gillett Co., Marshall, MI 49068). Results from preliminary experiments were used to determine inoculation procedures. Consequently, plants 2-3 wks of age were inoculated in the whorl with approximately 1.0 ml of 10^9 cells/ml suspension of *P. alboprecipitans* (PA 78-5). Blocks of tissue (5 mm x 5 mm) were sampled from whorl areas at 20, 40, 80, 160, and 320 min following inoculation. Prior to fixation, samples were exposed to high levels of carbon dioxide to close stomata so that bacteria would be captured in the stomata. Tissues were fixed for 3.0 hr at 22.0 C in a 3.0% gluteraldehyde 0.2 M cacodylate buffer (pH 7.3). In addition to freshly inoculated material, leaves with symptoms were also prepared for SEM examination. Diseased tissue was examined in order to become familiar with the appearance of bacteria in the leaf's interior. All samples were stored at 4 C. Samples were dehydrated in a series of 25, 50, 70, 90, 95, and 100% ethanol, followed by a series of 33.3, 50, 66.7, and 100% amyl acetate (diluent was 100% ethanol). Tissues were placed in a pressure chamber of a critical-point drier (DCP-1, Denton Vacuum, Inc., Cherry Hill, NJ 08034) and

infiltrated with liquid carbon dioxide until the amyl acetate was purged. Tissues were then critical-point dried. Samples were mounted on edge onto aluminum stubs and were coated with a gold-palladium alloy for 10 min in a Hummer I sputtering unit (Technics, Inc., Alexandria, VA 22313). Stomata, substomatal cavities, and mesophyll cells were examined at 20 KV with a scanning electron microscope (ETEC Omniscan, Hayward, CA 94551). See Hayat (25) for detailed SEM methods.

Infectivity titration. Ercolani (15) discussed the value of infectivity titration in the assessment of disease resistance in plants. A similar approach was used as a further assessment of the infection court. Sweet corn plants (cv. Gold Cup) were grown in a greenhouse. Corn plants 2-3 wks of age were either inoculated by pipet only in the whorl or by a spray mist on all plant surfaces. Plants inoculated with the spray mist were first treated with phenylmercuric acetate (50 ppm) in the whorl so that the whorl would not come in contact with viable bacteria. Inoculum (isolate PA 78-5 of *P. alboprecipitans*) was adjusted photometrically to 10^8 cells/ml, then was serially diluted to 10^2 cells/ml. Disease development in whorl inoculated plants for each dilution (10^8 - 10^2 cells/ml) was compared to disease development in spray-inoculated plants for the corresponding inoculum density. Two plants per plot with four replicates were assessed; therefore, data were evaluated on the response of a total of 56 plants (two plants/pot x four replicates x seven dilutions).

Protection of the whorl in the field. Experiments were conducted at Zellwood, FL on sweet corn, cv. Gold Cup.

Experimental plots were in a randomized complete block design with four replicates. Soil type was Lauderhill muck at pH 6.7 with approximately 90% organic matter. Plots consisted of five rows (20 plants/row) with one guard row on either side. Rows were spaced approximately 91 cm apart and plants were with an approximate 30 cm spacing in-the-row. Sweet corn plants in a mid-whorl growth stage (seventh leaf visible) were inoculated with a suspension of *P. alboprecipitans* (isolate PA 78-5) in June 1978. Inoculum was adjusted photometrically to 10^8 cells/ml. Bacteria were inoculated onto sweet corn plants with a two gallon compressed air sprayer. All plant surfaces were sprayed with the bacterial suspension until run-off occurred. Inoculations were performed between 8 and 10 p.m. because a higher proportion of plants were infected when inoculated in the evening (data from preliminary experiments). One hour prior to inoculation, test plants were treated with 5.0 ml of 50 ppm aqueous solution of phenylmercuric acetate (PMA). The bactericidal PMA was applied locally into the whorl with an automatic pipet. Approximately 1 wk after the day of inoculations, the seventh leaf from 20 different sweet corn plants was sampled from each plot. Leaves from four plants in each of five rows were sampled from the plants in the center of the plots. The seventh leaf was selected because PMA caused a chlorotic band across the tissue which was in the whorl at the time of inoculation. Each leaf was examined for per cent of leaf area damage due to BLBSRC. Measurements were made by the dot counting method (39). Not

only was total leaf damage compared between control and PMA treatment, but each leaf was divided into 10 cm zones so that each zone could be compared. The size of each leaf-zone was based on the size of the chlorotic band which identified the tissue in the whorl. Division of the leaf was initiated from the apical end and the leaves consisted of eight leaf-zones. Therefore, leaf-zone 1 would represent the first 10 cm of leaf tissue that occupied the whorl and so on until finally leaf-zone 8 (base of leaf) represented the final 10 cm or less of tissue that occupied the whorl. Leaf-zone 3 occupied the whorl area at time of inoculation as it was marked by the phytotoxic PMA. Finally, per cent damage by BLBSRC in each leaf-zone was measured by the dot counting method and damage in leaf-zones of control leaves was compared to damage in leaf zones of leaves from plants treated with PMA.

Protection of the whorl in the greenhouse. Additional experiments were conducted in a steam-heated greenhouse (30-35 C) in November through December of 1978. Sweet corn plants (cv. Gold Cup) were grown in a steam-sterilized potting mix (one part Canadian peat:one part perlite:three parts sandy loam) in clay pots (12.5 cm in diameter). Experimental design was randomized complete block with four replicates. Chemical, physical, and biological protection of the whorl was examined. Test plants were treated with either cotton plugs, 50 ppm PMA, 150 ppm of experimental bactericide Bay Sen 2213 (Mobay Chemical Co., Kansas City, MO 64141), 10^8 cells of *Klebsiella pneumoniae* (Schroeter) Trevisan/ml, 10^8 cells of *Erwinia herbicola* (Lohnis) Dye/ml (10). All treatments were applied only to whorls of

sweet corn plants 2-3 wks of age. A 10^8 cells/ml suspension of *P. alboprecipitans* (PA 78-5) was inoculated on to all corn plant surfaces following the whorl treatments. Inoculum was applied as a spray mist with an aerosol chromatography sprayer (Spray-tool 15-233, Fisher Scientific Co., Pittsburgh, PA 15230). Leaf samples were taken one week after inoculation. The fifth leaf was sampled as it was identified by a chlorotic band as being in the whorl at the time of treatments and inoculation. Each leaf was measured by the dot counting method for damage due to BLBSRC.

Analysis of potential control strategies

Bio-antagonism. In order to clarify if *K. pneumoniae* or *E. herbicola* have potential as bio-antagonists to *P. alboprecipitans*, a mixed culture technique described by Goodman (19) was utilized in this study. A streptomycin-resistant mutant of *P. alboprecipitans*, isolate PA 73-31R (18), was cultured with *K. pneumoniae* (KP 78-1) or *E. Herbicola* (EH 78-1). The initial concentration of each organism in the growth medium was determined to be 10^3 cells/ml. Mixed cultures were incubated in 100 ml of nutrient broth on a rotary shaker for 24 hr. Each treatment had three replicates. The same procedures were repeated except isolates of *K. pneumoniae* and *E. herbicola* were introduced with *P. alboprecipitans* in a ratio of 99:1.

After 24 hr, 0.05 ml samples from each culture were serially diluted by a micro-titer dilution system. Populations for each organism were calculated from two replicates of dilutions on nutrient agar. An alternative method was necessary to detect PA 73-31R. The population of the

streptomycin-resistant mutant was determined by the dilution plate method on nutrient agar which had been supplemented with streptomycin to a final concentration of 400 ppm.

Detection of resistant varieties. Varieties of sweet corn resistant to BLBSRC have been reported (54). Four of these varieties (Apache, Merit, Gold Cup, and Bonanza), which vary in the degree of resistance to BLBSRC, were selected for experimentation. The mean stomatal index values were calculated for each variety. The index value was calculated by dividing the number of stomata in a field of vision (40x) with the number of stomata plus the number of epidermal cells in a field of vision. The quotient was then multiplied by 100 to give the index value. The index values were calculated from four replicates of household vinyl-cement (Sears, Roebuck and Co., Chicago, IL 60607) impressions of leaf surfaces. In addition to known responses in the field (54), Apache, Merit, Gold Cup, and Bonanza were evaluated for disease resistance to BLBSRC in a greenhouse. Plants 2-3 wks of age were spray-mist inoculated with 10^8 cells of PA 117/ml. Four replicates were evaluated for per cent leaf damage by the dot counting method. The mean index value and per cent leaf damage of the four varieties were analyzed by regression-correlation to determine if index values could be used to predict a varietal response to BLBSRC.

An alternate method to detect resistant varieties was attempted. The movement of *S. marcescens* in different varieties was recorded. Approximately 0.5-1.0 ml of 10^8 cells of the red-pigmented *S. marcescens*/ml was applied to the whorl of

Gold Cup, Merit, Apache, and Bonanza sweet corn varieties. Plants 2-3 wks of age were subjected to conditions of high relative humidity (flooded greenhouse floor) and intensive watering. The leaf in the whorl from each variety was sampled after a 5-hr exposure to the bacteria. Each leaf was aseptically divided into lower, middle, and upper segments and then crushed in tubes of sterile saline. A loopful from each tube was streaked onto nutrient agar and incubated at 30 C. Plates were examined for presence of *S. marcescens* after a 36-hr incubation period. Detection of the bacteria in upper zones was analyzed in relation to varietal response to BLBSRC. Results were based on four replicates.

Stomatal manipulation. Sweet corn plants (cv. Gold Cup) 2-3 wks of age were sealed in glass chromatography tanks (30 cm x 30 cm x 60 cm). Tank bottoms were flooded with 1ℓ of 2M KOH to reduce the CO_2 content of the enclosed atmosphere. Potted plants (12.5 cm in diameter) were placed on petri plates to avoid absorption of the KOH and plants were subjected to a low CO_2 atmosphere for 48 hr. Plants in the tanks were subjected to continuous light (6500 lux) and a 28 C temperature in a CEL 4-4 controlled environment chamber. Test plants were sprayed with either 10^{-4}M solution of phenylmercuric acetate (PMA) or 10^{-4}M solution of abscisic acid (ABA). After a 1 hr exposure to either ABA or PMA, leaf impressions were made of corn leaf surfaces. The leaf impression technique (68) was modified as leaf impressions were made with household vinyl cement. One hundred stomatal apertures were measured per plant in four replicates. Corn plants with open stomata

(48 hr KOH) and closed stomata (48 hr KOH + 1 hr ABA) were inoculated with 10^8 cells of PA 117/ml. Inoculum was applied as a spray-mist with an aerosol chromatography sprayer. After symptom development (72-96 hr), plants were scored for BLBSRC as per cent leaf damage.

RESULTS

Evidence for the identification of the infection court

Analysis of whorl water. *Pseudomonas alboprecipitans* grew well in dewand guttation water that had been collected from the whorl of sweet corn plants. Bacteria reproduced and increased one hundred-thousand fold in filter-sterile whorl water (SWW) (Table 1). The respiration rate of isolate PA 117 in SWW was slightly greater than the respiration rate when cultured in nutrient broth and greatly exceeded the rate in buffered saline (Fig. 1). Crude analysis of SWW fractions resulted in the detection of sugars, organic acids, and amino acids. The identities of the compounds were not determined beyond the general class of the compound. The inconsistencies in Rf values of standards and unknowns as well as the limited sample size prevented a detailed analysis. However, it was concluded that SWW from sweet corn was not inhibitory to PA 117. Furthermore, it was concluded that essential compounds for microbial growth were present in whorl water.

Movement of markers. Additional evidence for the identification of the infection court was obtained with the introduction of markers to the corn plant. After introducing a fluorescent dye (fluoroscein) into the whorl of corn plants, the dye moved upward into the leaves and produced a striped pattern similar to lesions caused by *P. alboprecipitans*. In

Table 1. Replication of *Pseudomonas alboprecipitans* (PA 117) when introduced into whorl water collected from sweet corn, cv. Gold Cup.

Sample of Whorl Water	Population of PA 117[1] after 48 hr
a	3.4×10^8 cells/ml
b	4.0×10^8 cells/ml
c	2.5×10^8 cells/ml
	$\bar{x} = 3.3 \times 10^8$ cells/ml

[1]Initial population was 5×10^3 cells/ml.

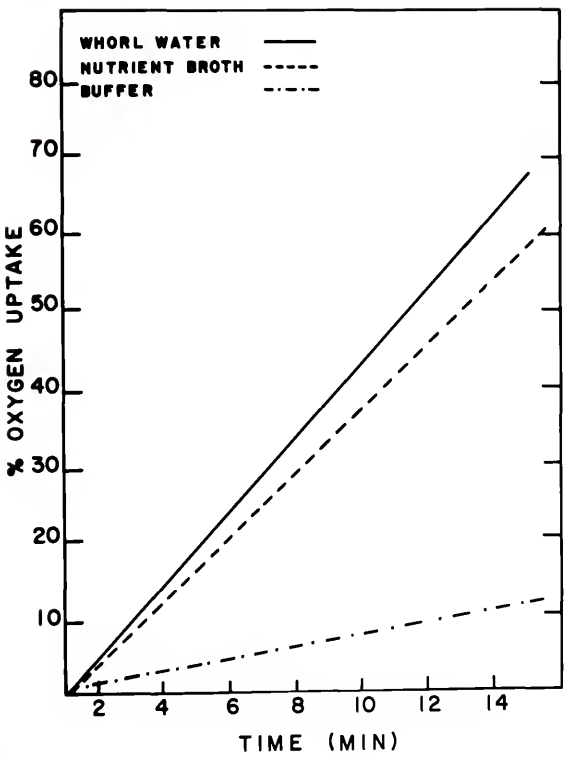


Fig. 1. Rate of respiration of *Pseudomonas alboprecipitans* in whorl water from sweet corn, nutrient broth and phosphate-buffered 0.85% saline (pH 7.0). Figure represents the mean of two replications.

addition, evidence that bacteria moved into leaves was also obtained. The movement of *S. marcescens* from the whorl into and up the leaf was monitored over time (Table 2). Regardless of the length of time exposed to bacteria, all samples from leaf-zone 1 contained the marker. However, there was a trend of greater movement being correlated with increased exposure times. It was significant that after a 24-hr exposure, bacteria moved six leaf-zones or 12 cm above the whorl. Although data were recorded as presence or absence in a particular zone, there also was a slight difference in amount of bacterial growth between zones and between exposure times. The longer exposure times and zones closest to the whorl contained the heaviest growth, indicating that a gradient of bacteria moved up the leaf over time. Therefore, bacteria or dye applied to the whorl was capable of moving significant distances into the leaf. The movement of bacteria was probably similar to that of the dye, in that it was confined to the intercellular spaces between major vascular bundles.

Autoradiography. Evidence for stomatal ingress of *P. alboprecipitans* in whorl tissue was obtained through the stripping film autoradiography technique. Distribution of the ^{14}C label in sectioned tissue was concentrated in lesion areas as a result of glucose-^{14}C accumulation in PA 78-5 (Fig. 2). In addition, the labeled bacteria aggregated around stomata and sub-stomatal chambers in plants whose whorls were exposed to the bacteria for only 6 hr (Figs. 3 and 4). Clusters of labeled bacteria often appeared in the same vicinity in 10 μm sequential sections (Figs. 3 and 4). The density of exposed

Table 2. Time-course study of *Serratia marcescens* movement in leaves after bacteria were applied to the whorl of sweet corn plants.

Exposure Time	Detection of Bacteria					
	Leaf-zones[1]					
	1	2	3	4	5	6
40 min	++++[2]	+++-	++--	----	----	----
80 min	++++	+++-	+++-	++--	+---	----
160 min	++++	++++	+++-	----	----	----
320 min	++++	++++	+++-	+++-	----	----
24 hr	++++	++++	++++	+++-	+++-	+---

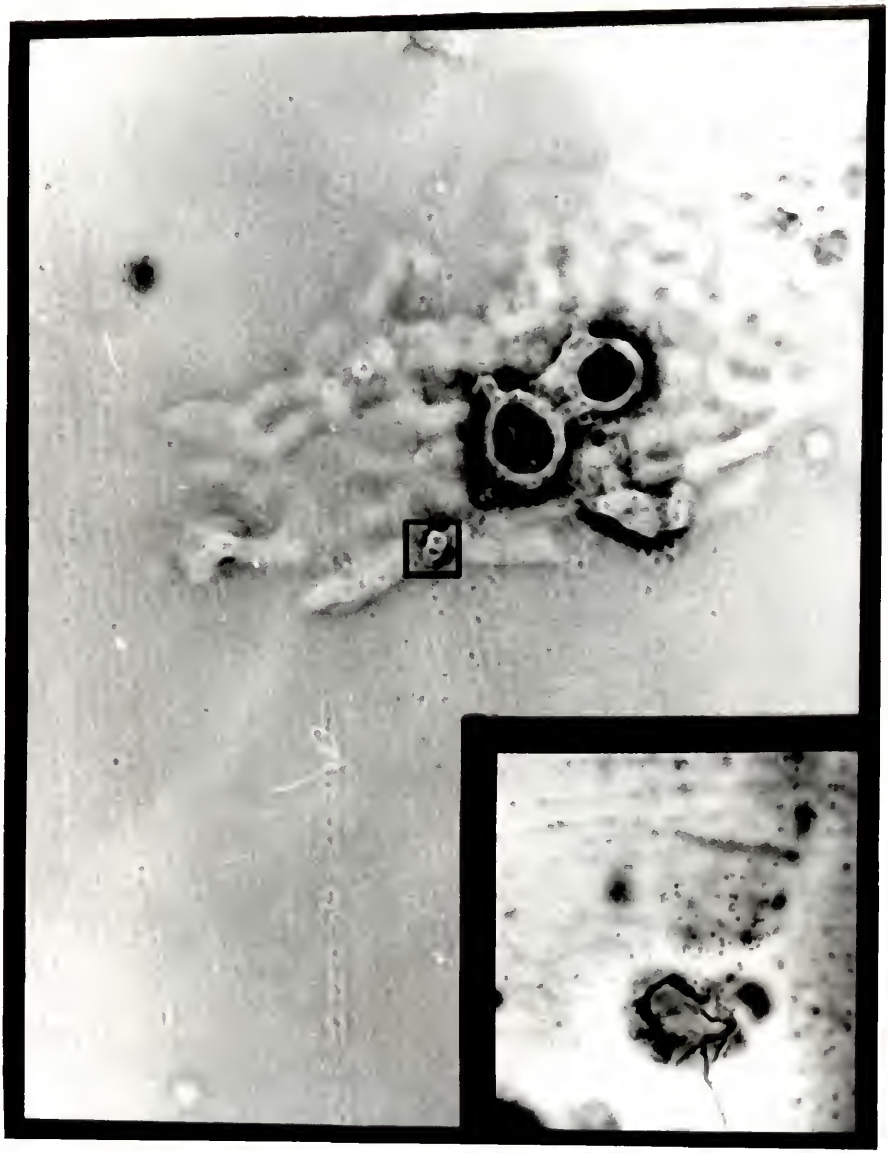
[1]Top of whorl zone was designated as base-line 0, each leaf-zone represents sections that were sampled at 2.0 cm intervals in an apical direction from the whorl.

[2]Data represents recovery from 4 replicates (i.e. ++-- means that 2 out of 4 replicates contained *S. marcescens*).

Fig. 2. Autoradiograph of *Pseudomonas albopre-cipitans* labeled with glucose-^{14}C. Exposed silver grains are localized in lesion area on sweet corn leaf. Cross section of leaf (unstained) is approximately x2500.

Fig. 3. Autoradiograph of *Pseudomonas albo-precipitans* labeled with glucose-^{14}C. Cross-sectional view of leaf (unstained) is approximately x200. Leaf was sampled from the whorl after a 6-hr exposure to labeled bacteria. Lower inset shows higher magnification (x1000) of boxed area on leaf cross-section. Exposed silver grains are localized in stomatal area.

Fig. 4. Autoradiograph of 10 μm cross-section of a corn leaf that had been inoculated with ^{14}C labeled *Pseudomonas alboprecipitans* (x150). Lower inset shows higher magnification (x1000) of boxed area on leaf cross-section. Boxed area is in similar position in leaf as boxed area in previous section. Exposed silver grains are localized in the stomatal area.



silver grains was significantly greater ($P=0.01$) in target areas (lesion or stomata) than in background tissue (Table 3).

Scanning electron microscopy. Additional evidence for stomatal ingress in whorl tissue was obtained with scanning electron microscopy (SEM). Examination of diseased tissue showed that lesions caused by *P. alboprecipitans* were vein limited and consisted of severely collapsed tissue (Fig. 5). The tearing away of tissue from the vascular bundle due to the severe collapse of cells in necrotic areas offers an explanation of why shredding of leaves is a typical symptom in BLBSRC. Corn leaves have vascular bundles that are parallel and with limited cross support. Therefore, the tearing of tissue adjacent to the vascular bundle causes the leaf to be vulnerable to wind damage. Intercellular spaces of the lesion area were occupied by numerous rod-shaped bacteria (Fig. 6). Bacteria were present as isolated cells or clumped in microcolonies. The rod-shaped morphology and great number of particles observed were reasonable criteria to assume that the organism was *P. alboprecipitans*.

Scanning electron micrographs of cross-sections of corn leaves of noninoculated control plants did not contain any rod-shaped particles (Figs. 7 and 8). In addition, SEM micrographs of control leaves revealed that stomata were open in the whorl at time of inoculation as well as being free of rod-shaped particles (Figs. 9-11). Furthermore, because tissues came from whorl areas that were flooded with water, stomata must have been open even when submerged.

Table 3. Statistical analysis of autoradiographic experiments. Density of exposed silver grains in target areas (lesions and stomata suspected to contain *Pseudomonas alboprecipitans*) compared to density of exposed silver grains in background areas.

Sample Site	Developed Silver Grains per 0.025 mm^2	Calculated t-value
Lesion	233[2]	12.97**
Background	58	
Stomata	128[3]	7.02**
Background	25	

t0.01 7df = 3.49, t0.01 9df = 3.25

[1]Stomatal samples were from plants exposed to PA 78-5 for 6 hr.

[2]Mean of eight sample sites.

[3]Mean of ten sample sites.

**Significant (P=0.01) as determined by student's t-test.

Fig. 5. SEM micrograph of cross-sectional view of bacterial leaf blight and stalk rot lesion in sweet corn leaf (x1000). Tissue in the lesion is collapsed and lesion is limited by vascular bundles with a sclerenchyma sheath. Collapse of lesion tore tissue away from the vascular bundle.

Fir. 6. SEM micrograph of cross-sectional view of bacterial leaf blight and stalk rot lesion in sweet corn leaf (x10,000). Intercellular spaces are congested with isolated rod-shaped bacteria and micro-colonies of *Pseudomonas alboprecipitans*.

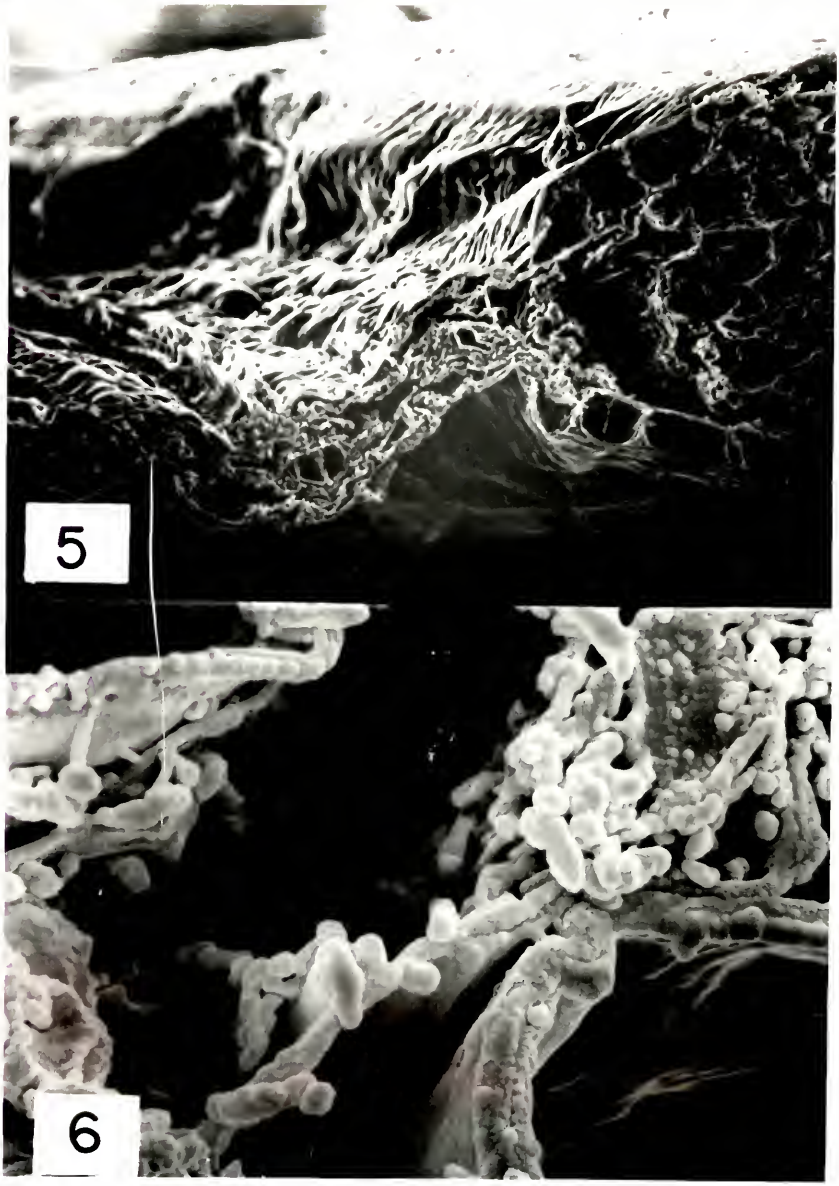

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Fig. 7. SEM micrograph of cross-sectional view of stoma and sub-stomatal chamber in control corn-leaf (x1160). Sub-stomatal chamber is devoid of any rod-shaped bacteria-like structures.

Fig. 8. SEM micrograph of cross-sectional view of stoma and sub-stomatal chamber in control corn-leaf (x4000). Stoma and sub-stomatal chamber are devoid of bacteria. View is of tissue sampled from whorl, note that stoma is open.

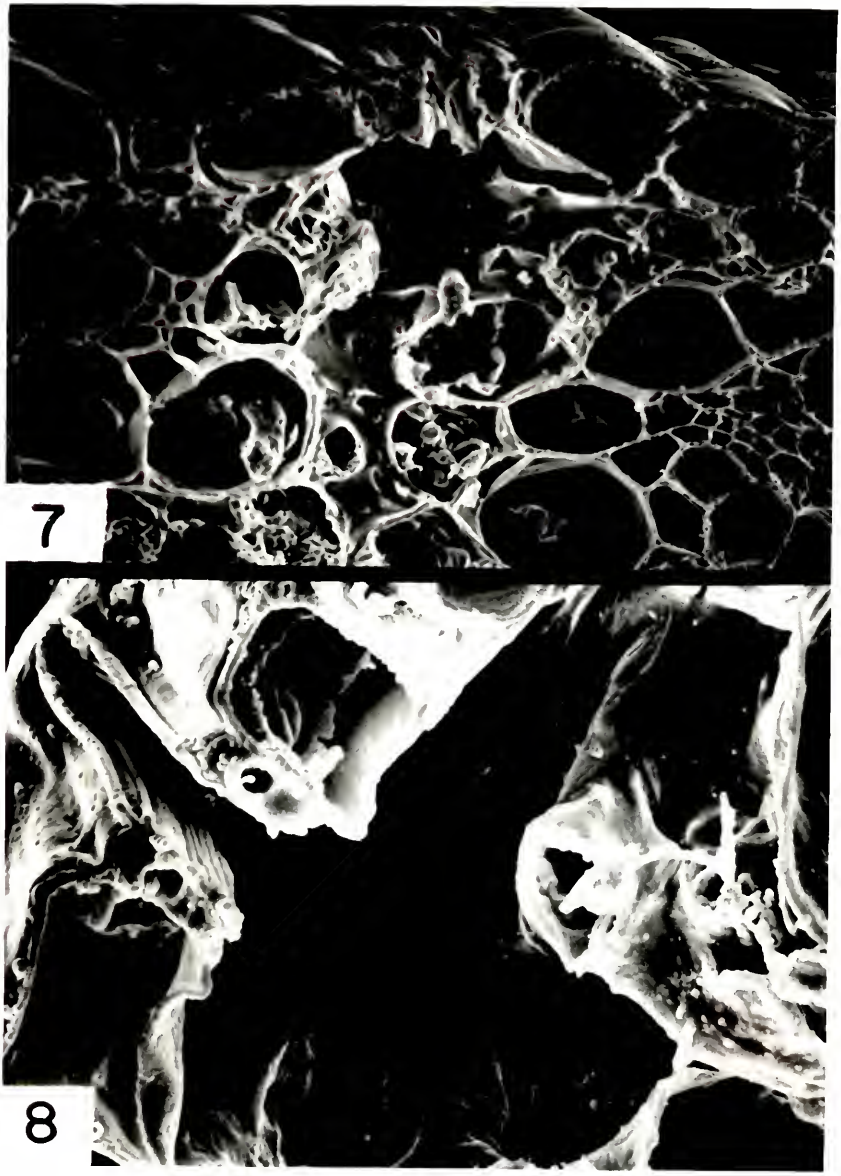
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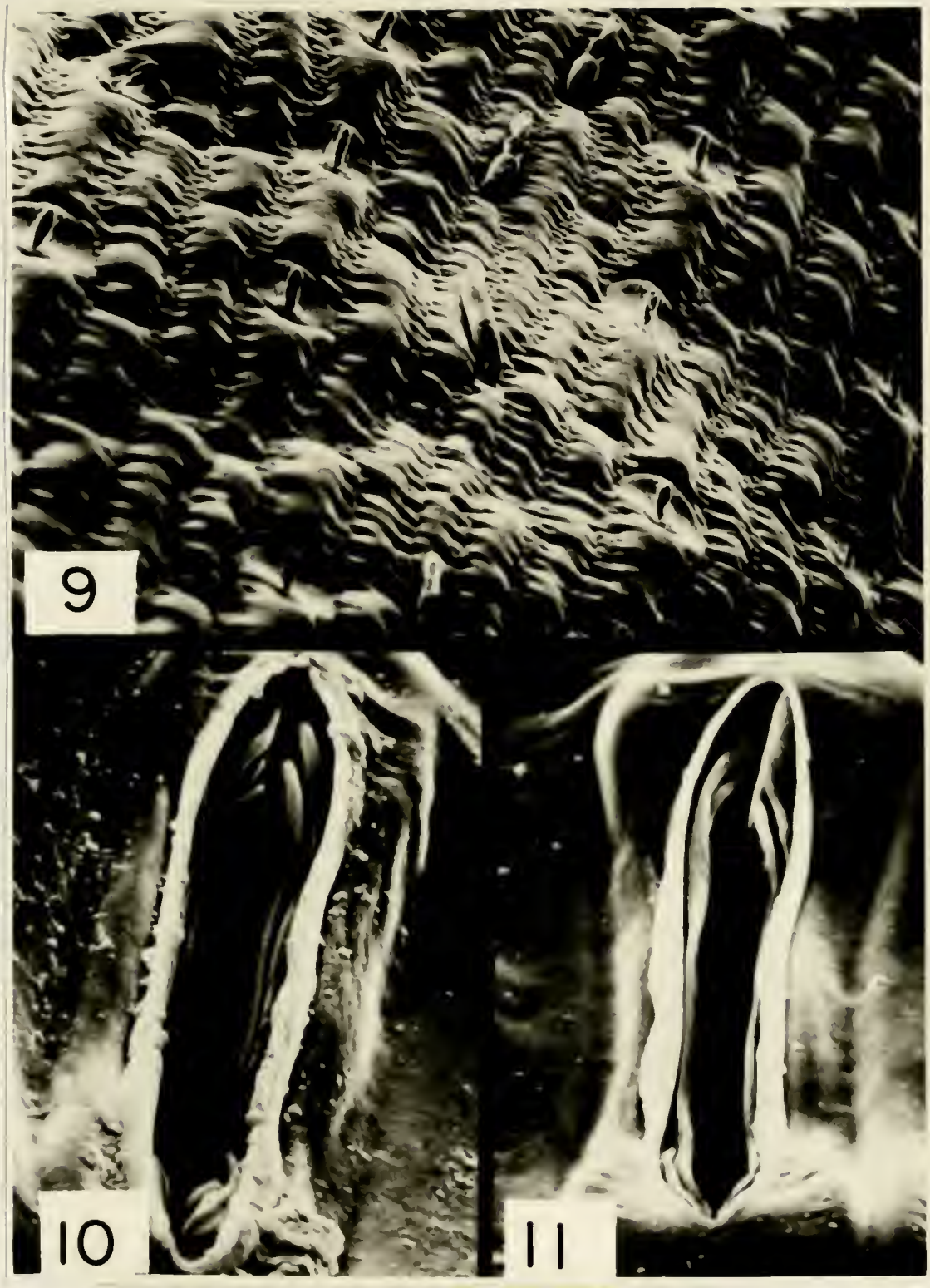
Fig. 9. SEM micrograph of the surface of control leaf which did not receive stomatal closing treatment (x520). Corn tissue sampled from whorl area had stomata open even though the whorl contained free water.

Fig. 10. SEM micrograph of the surface of open stoma on control leaf (x4970). Bacteria were not observed in stoma or on the surface of the surrounding area.

Fig. 11. SEM micrograph of the surface of open stoma on control leaf (x4800). Bacteria were not observed in stoma or on the surface of the surrounding area. Stoma was open even though the sample came from the whorl area which was flooded with free water.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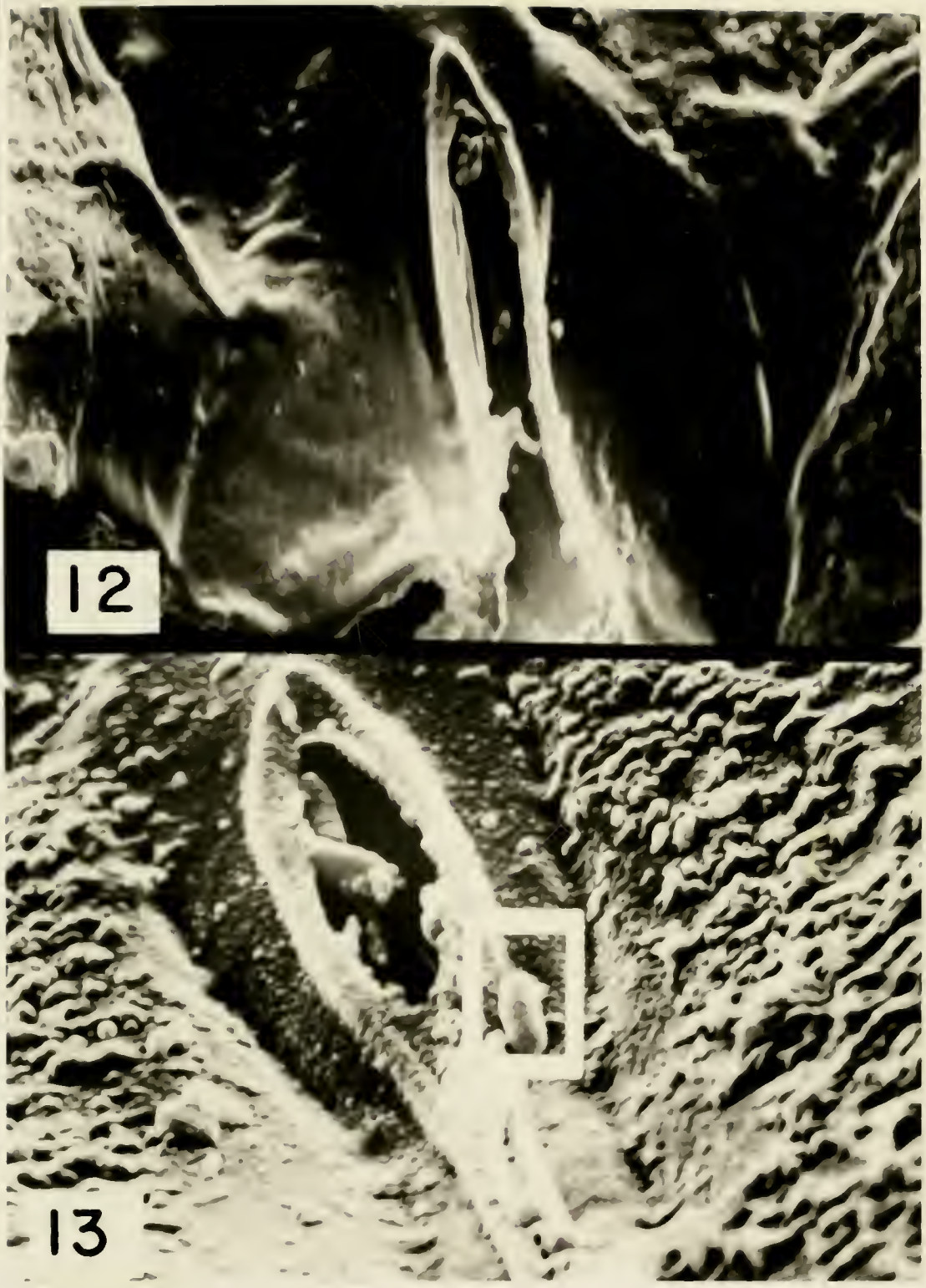
Bacteria (rod-shaped particles, mean size of 0.57 x 1.5 μm) were observed in stomata of corn plants that were exposed to *P. alboprecipitans* (Figs. 12-15). Observations of bacteria in stomata ocurred most frequently when plants were exposed to the inoculum for a period of 320 min (Figs. 12-14). Only on one occasion were bacteria observed in stomata of plants that were exposed to inoculum for 160 min (Fig. 15). Corn stomata bulge upon closing which creates a basin at both ends (Figs. 13 and 14). These small depressions were frequently filled with bacteria. It was hypothesized that if the stomata were open then bacteria would have passed through into the intercellular spaces. Therefore, closing the stomata prior to SEM fixation, assured the observation of bacteria caught in a stoma. Otherwise bacteria would have passed through the stoma. In support of that hypothesis, bacteria were observed in substomatal chambers of corn leaves exposed to *P. alboprecipitans* for 320 min (Figs. 16 and 17). It was considered highly improbable that bacteria could have been in sub-stomatal chambers by any other means than having entered via the stomata. Bacteria were not observed in any location of leaf cross-sections other than in substomatal chambers.

Infectivity titration. The evidence supports the idea that the whorl can be an infection court for *P. alboprecipitans*. However, additional evidence was accumulated to determine if whorl areas were more favorable for infection than other plant sites. The comparison of whorl inoculations against inoculations of the total plant (excluding the whorl) supported the whorl as the most favorable site (Table 4). When the total

Fig. 12. SEM micrograph of surface view of a corn stoma (x5000). Tissue was sampled from the whorl area of a corn plant exposed to PA 78-5 for 320 min. Rod-shaped bacteria-like particles (0.5 x 1.0 μm) are present in partially closed stoma.

Fig. 13. SEM micrograph of surface view of a corn stoma (x10,000). Sample was from the whorl area of a corn plant exposed to PA 78-5 for 320 min. Rod-shaped bacteria (0.5 x 1.5 μm) are lodged in a basin formed by guard cells at the end of a closed stoma. Boxed area is a bacterial cell on the surface of a guard cell.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13

Fig. 14. SEM micrograph of surface view of a corn stoma (x13,333). Sample was from the whorl area of a corn plant exposed to PA 78-5 for 320 min. Rod-shaped bacteria (0.5 x 1.0 μm to 0.5 x 1.5 μm) surround and are in a basin formed by a closed stoma.

Fig. 15. SEM micrograph of surface view of a corn stoma. Sample was from the whorl area of a corn plant exposed to PA 78-5 for 160 min.
(a) Rod-shaped bacteria (0.5 x 1.0 μm) are deep in the stoma (x8260).
(b) Outlined boxed area in 15a is enlarged to show single bacterial cell in stoma (x35,000).


14
15 a
15 b

Fig. 16. SEM micrographs of cross-sectional view of a corn leaf that was exposed to PA 78-5 for 320 min.
(a) Sub-stomatal chamber below guard cells (x2955).
(b) Enlargement (x7500) of sub-stomatal area that is outlined in 16a. Note the presence of rod-shaped bacteria (0.5 μm x 1.6 μm) lodged against cell walls in the intercellular space.

Fig. 17. SEM micrographs of cross-sectional view of a corn leaf that was exposed to PA 78-5 for 320 min.
(a) Intercellular space between upper and lower stomata (x2000).
(b) Enlargement (x14,000) of sub-stomatal area that is outlined in 17a. Note the presence of rod-shaped bacteria (0.5 μm x 1.5 μm) in the intercellular space.

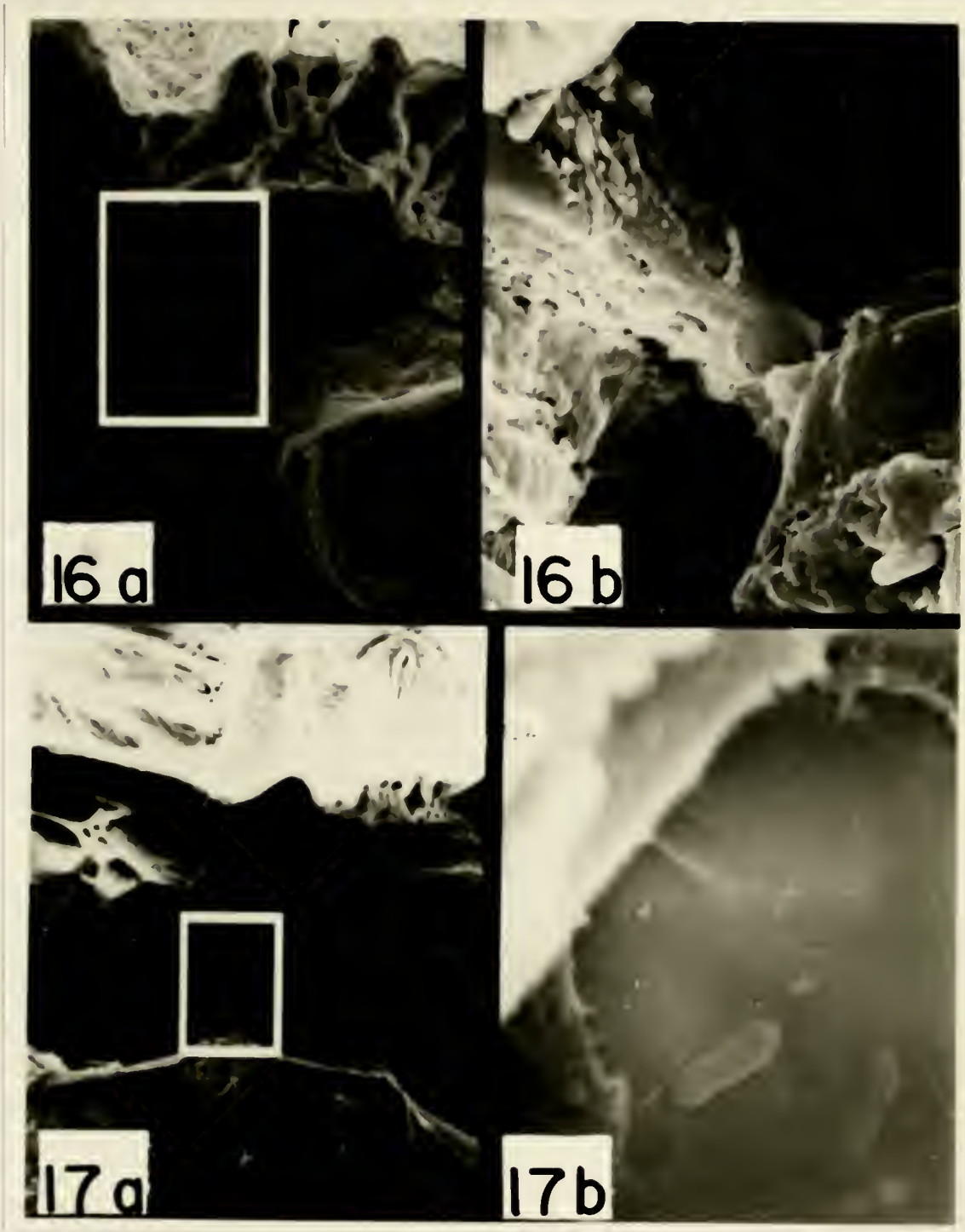
16 a
16 b
17 a
17 b

Table 4. Comparison of whorl vs spray inoculation by infectivity titration, efficiency based upon lesion presence[1] on sweet corn leaves that were exposed to *Pseudomonas alboprecipitans*.

Inoculation Method	Lesion Presence						
	Inoculum Density[2]						
	10^8	10^7	10^6	10^5	10^4	10^3	10^2
Spray mist	+	+	+	-	-	-	-
Whorl	+	+	+	+	+	+	+

[1]Development of lesions at 10^6 bacterial cells/ml and below was uniform, therefore data was compiled as presence (+) or absence (-).

[2]Inoculum density = cells/ml.

plant was sprayed with bacteria, lesions developed only at inoculum densities of 10^6 - 10^8 cells/ml. However, bacteria pipetted into the whorl resulted in disease at inoculum levels as low as 10^2 cells/ml. The amount of disease was reduced at the lower inoculum levels as only one lesion developed on plants exposed to 10^2 - 10^6 cells/ml.

Protection of the whorl in the field. Additional data were collected in a field study to further elucidate the site(s) of the infection court. Plants treated with PMA in the whorl developed a chlorotic band across the emerged leaf which corresponded to the leaf area which was in contact with the bactericide. The sample leaf possessed a 10-cm chlorotic zone between 20-30 cm from the leaf tip. This area was designated as leaf-zone 3 and it was the leaf-tissue in the whorl at time of inoculation. Plants treated with a local application of PMA had significantly less disease than control plants (P=0.05). Lesions first appeared in leaf-zone 3 of control leaves. Disease steadily increased in severity until leaf-zone 7 (Fig. 18). Plants treated with PMA first developed lesions in leaf-zone 4, but disease did not begin to increase until leaf-zone 6 (Fig. 18). Therefore, the PMA treatment prevented the increase of disease in 2-3 leaf-zones. Corn leaves grow from a basal leaf meristem; therefore apical leaf-zones emerge from the whorl before basal zones do. Consequently, leaf-zones can be viewed in terms of time when the tissue occupied the whorl area. Corn grows rapidly, and the extension of 20-30 cm of leaf area requires approximately 48 hr. Therefore, the duration of protection by PMA of 2-3 leaf-

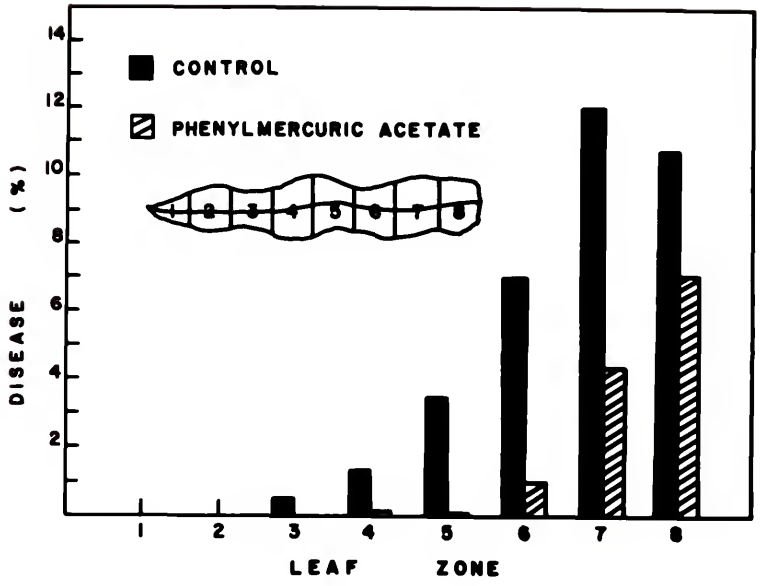

Fig. 18. Per cent leaf area damaged by *Pseudomonas alboprecipitans* in eight leaf-zones (10 cm each) of control leaves compared to disease in corn leaves treated with a local application of phenylmercuric acetate (PMA) in the whorl. Leaf-zone 3 was in the whorl at time of treatment.

zones delayed disease development by only 48 hr. Finally, it was observed that leaves on the lower portion of the plant did not develop symptoms. The lower leaves were unfurled and extended at time of inoculation.

Protection of the whorl in the greenhouse. The protection of the plant by treatments in the whorl was not as definitive in the greenhouse as in the field. Treatment with PMA significantly reduced disease ($P=0.05$) in one greenhouse test (Table 5). However, in a second experiment, treatment with PMA did not differ from controls (Table 6). Per cent disease severity was questioned as the best analytical procedure, as variation occurred within each treatment. Therefore, position of lesion development on the corn leaf was used to compare the effects of whorl treatments. Leaf number 5 displayed most of the symptoms and it was in the whorl at time of inoculation and treatments. Therefore, leaf 5 was sampled and the base of the leaf was designated as the zero reference point. Leaves from PMA-treated plants possessed a chlorotic band 57 mm in width which developed between 286-343 mm from the base of the leaf (Table 7). This was the leaf-tissue in the whorl at the time of inoculation and treatment. Lesions developed at 333 mm and extended to near the apical tip of the leaf (680) in plants treated with PMA (Table 7). Lesion area in the fifth leaf of control plants streaked from 156 mm to 717 mm (Table 7). Therefore, lesions occurred above the whorl in PMA-treated and control plants which demonstrated that tissues above the whorl could be a site for infection. However, lesions in control plants also developed well

Table 5. Efficacy of chemical and biological treatments for control of bacterial leaf blight and stalk rot of corn, when treatments were applied locally to corn-plant whorls.

Treatment	Disease Index[1]
Control	11.05 a[2]
Klebsiella pneumoniae	9.23 a b
Serratia marcescens	5.65 a b c
Erwinia herbicola	4.41 b c
PMA (phenylmercuric acetate)	1.57 c

[1]Per cent area damaged of leaves in the whorl during inoculation.

[2]Mean disease index values not followed by the same letter are significantly different ($P=0.05$) as determined by analysis of variance and Duncan's New Multiple Range Test.

Table 6. Efficacy of chemical, biological, and physical treatments for control of bacterial leaf blight and stalk rot of corn, when treatments were applied locally to corn-plant whorls.

Treatment	Disease Index[1]
Control	8.92 a[2]
Bay Sen 2213	8.34 a
Cotton plug	8.32 a
Klebsiella pneumoniae	7.02 a
PMA (phenylmercuric acetate)	6.60 a b
Erwinia herbicola	3.39 b

[1]Per cent area damaged of leaves in the whorl during inoculation.

[2]Mean disease index values not followed by the same letter are significantly different ($P=0.05$) as determined by analysis of variance and Duncan's New Multiple Range Test.

Table 7. Effect of local whorl treatments on the distribution of bacterial leaf blight and stalk rot lesions on sweet corn leaves.[1]

Treatment	Position[2] of Basal End of Lesion
PMA (phenylmercuric acetate)	333 mm a[3,4]
Erwinia herbicola	198 mm b
Control	156 mm b

$\bar{x}$ whorl zone = 286-343 mm

[1]Data represent leaf 5, as fifth leaf was in the whorl during inoculation. Data represent mean of four replications.

[2]Position marks point on leaf in mm from the base of the leaf, as base end was designated 0.

[3]Values not followed by the same letter are significantly different ($P=0.05$) as determined by analysis of variance and Duncan's New Multiple Range Test.

[4]Apical end of lesion position (680-720 mm) was not significantly different ($P=0.05$) among treatments.

below the whorl zone, which showed that a local treatment of PMA in the whorl did affect disease development. In contrast, leaves emerged at the time of inoculation remained healthy regardless of treatment. Therefore, it was concluded from this and previous experiments that tissues in or not far from the whorl were the most favorable sites for infection to occur.

Analysis of potential control strategies

Protection of corn against BLBSRC by whorl treatments with bactericide was effective, but was proved to be of short duration. Therefore, other control strategies were examined for their applicability to BLBSRC. The efficacy of chemical, biological and physical treatments of the whorl were evaluated. Protection of the whorl with the bactericide PMA was reported above; however, treatment with the experimental bactericide Bay Sen 2213 had no effect against BLBSRC (Table 6). In addition, the physical blockage of the whorl with cotton plugs failed to affect disease development in the plant (Table 6).

Bio-antagonism. The whorl as an infection court was an ideal system to experiment with bio-control. The establishment of a regenerating antagonistic microorganism in the whorl was attempted. *Erwinia herbicola* appeared the most effective bio-control agent of the organisms tested (Tables 5 and 6). However, the discrepancy between separate tests warranted a different method of analysis. Therefore, position of lesion development on the corn leaf was used to analyze the effects

produced by *E. herbicola*. The fifth leaf displayed most of the symptoms and it was in the whorl at time of inoculation and treatment. Therefore leaf 5 was sampled and the base of the leaf was designated as the zero reference point. The whorl area was determined to be the zone 286-343 mm from the base of the leaf. Lesions in control leaves developed from 156 mm to 717 mm (Table 7). Lesions in leaves from plants treated with *E. herbicola* developed from 198 mm and extended to 720 mm. There was no significant difference ($P=0.05$) in lesion distribution by a treatment with *E. herbicola* (Table 7). Furthermore, incubation of mixed cultures of *P. alboprecipitans* and *E. herbicola* provided no evidence of an antagonistic effect by *E. herbicola* (Table 8). Detection of a streptomycin-resistant mutant of *P. alboprecipitans* (PA 73-31R) mixed with *E. herbicola* was difficult when incubated on nutrient agar (Table 8). However, *E. herbicola* was eliminated by incubation of samples on nutrient agar which had been supplemented with 400 ppm streptomycin. The population of PA 73-31R on streptomycin-nutrient agar was calculated to be at least 10^8 cells/ml (Table 8). Similar results were found when *E. herbicola* was introduced at a substantially higher proportion (99:1) than PA 73-31R. Upon incubation and recovery on streptomycin-nutrient agar, at least 10^6 cells of *P. alboprecipitans*/ml were present. This was a population that could incite disease under the proper conditions (Table 4). Therefore, the bio-control capabilities of *E. herbicola* for BLBSRC were minimal at best. The use of an antibiotic resistant mutant was valuable for the analysis of bacterial populations in mixed cultures.

Table 8. Survival of a streptomycin-resistant mutant of *Pseudomonas alboprecipitans* (PA 73-31R) when incubated with either *Klebsiella pneumoniae* (KP 78-1) or *Erwinia herbicola* (EH 78-1).

Isolate Pair	Population Density	
	Nutrient Agar (NA)	NA+400ppm Streptomycin
PA 73-31R	——	8.0×10^6 cells/ml
KP 78-1	9.0×10^8 cells/ml	——
PA 73-31R	2.2×10^6 cells/ml[1]	1.4×10^8 cells/ml
EH 78-1	3.8×10^8 cells/ml	——

[1]Estimate based on one colony observed at 10^{-6} dilution.

Detection of resistant varieties. Although application of chemicals or microorganisms to the whorl did not adequately protect corn against BLBSRC, characteristics of the infection site may have a relation to other control practices. Stomata in whorl tissue were established as portals of entry for *P. alboprecipitans*; therefore the stomatal index (number of stomata per number of epidermal cells + stomata x 100) was examined as a possible criterion for identifying resistant varieties. The variety Gold Cup had a mean stomatal index (SI) value of 15 which exceeded values for the varieties Merit, Apache, and Bonanza (Table 9). An SI value of 15 was equivalent to 18 stomata per 100 epidermal cells. Bonanza had the lowest SI value of 10.1 which was equivalent to 11 stomata per 100 epidermal cells. Apache was the most susceptible variety and Bonanza the most resistant under greenhouse conditions (Table 10). These responses were similar to those reported by Sumner and Schaad (54) for field conditions. However, there was no apparent correlation between SI and resistance to BLBSRC as the correlation coefficient (r) was only -0.02 for the prediction equation $y=16.82 - 0.125x$.

Another method for the identification of resistant varieties was attempted by monitoring the movement of a marker (*S. marcescens*) in different varieties. After the application of *S. marcescens* into the whorl, its movement up the leaf did not relate to varietal response to BLBSRC. Under greenhouse conditions, the greatest movement of the marker occurred in Bonanza (the most resistant variety).

Table 9. Stomatal index values of upper leaf surfaces of four sweet corn varieties.

Variety	Stomatal Index Value (SI)[1] Replicates					
	1	2	3	4	$\bar{x}$	
Gold Cup	15	15.5	16.4	13.1	15	a[2]
Apache	12.2	13.8	12.2	12.9	12.8	b
Merit	13.8	13.9	12.1	10.1	12.5	b
Bonanza	8.7	11.2	10.0	10.4	10.1	c

[1]SI = #stomata/#stomata + #epidermal cells x 100; based on 25 samples from each replicate.

[2]Values not followed by the same letter are significantly different ($P=0.05$) as determined by analysis of variance and Duncan's New Multiple Range Test.

Table 10. Per cent leaf damage in sweet corn varieties that were inoculated with *Pseudomonas alboprecipitans*.

Variety	Reported Response to *P. alboprecipitans*[1]	Per cent leaf damage					
		Replicates					
		1	2	3	4	$\bar{x}$	
Apache	highly susceptible	42	44	40.7	35.8	40.6	a[2]
Merit	highly susceptible	15.7	0	35.5	20	17.8	b
Gold Cup	moderately susceptible	0	0	0	10.4	2.6	c
Bonanza	resistant	0	0	0	0	0	c

[1]Sumner and Schaad (54).

[2]Mean per cent damage values not followed by the same letter are significantly different ($P=0.05$) as determined by analysis of variance and Duncan's New Multiple Range Test.

Stomatal manipulation. An alternative control measure was attempted by the manipulation of stomata. Corn plants have stomata which are sensitive to the presence of carbon dioxide (CO_2). Low levels of CO_2 will close most corn stomata. Therefore, to demonstrate that stomata could be manipulated, treatments were compared to corn plants maintained in a low CO_2 atmosphere for 48 hr. Plants in a low CO_2 atmosphere possessed open stomata (Fig. 19) whereas similar plants treated with 10^{-4} M abscisic acid (ABA) had many of their stomata closed (Fig. 20). The use of a fast-drying vinyl cement was essential in determining stomatal apertures. The usual method of silicon impressions (68) was not satisfactory for obtaining accurate leaf impressions. The curing of silicon closed stomata, which was probably due to the length of time required for silicon curing (up to 30 min). The vinyl cement produced accurate impressions (Fig. 19 and 20) and it had the advantage of being fast drying (a matter of seconds), thereby capturing the stomatal aperture before there was a change in size. Therefore, treatments with ABA and PMA could be quantitatively assessed (Table 11). Although ABA and PMA inhibited stomatal opening in corn leaves, a range of responses between open and closed did occur (Table 11). Due to the range between open and closed states, treatment with stomatal inhibitors did not significantly reduce ($P=0.05$) disease development (Table 12).

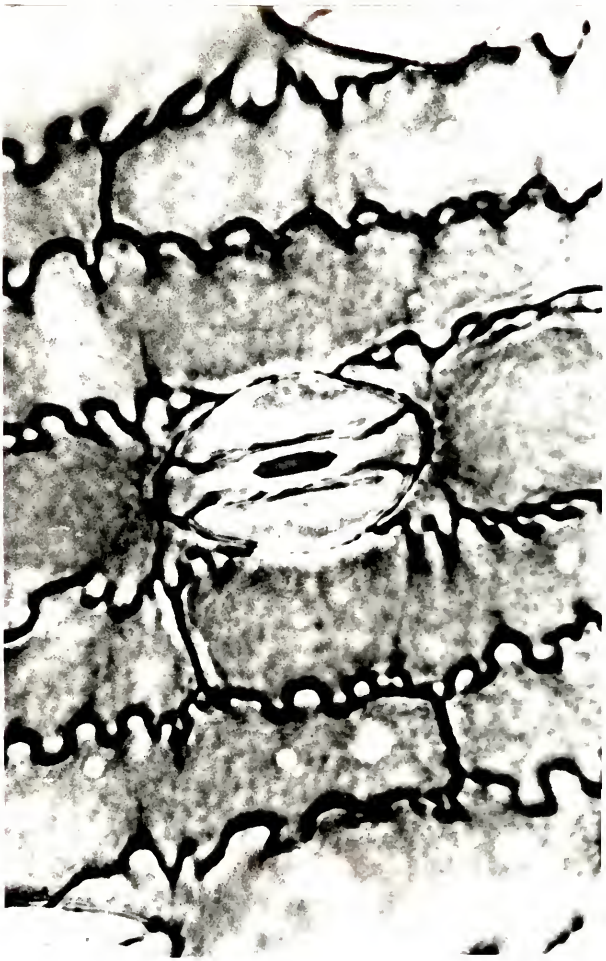

Fig. 19. Leaf impression of open stoma (5 μm across center of aperture) from corn leaf exposed to a low CO_2 atmosphere for 48 hr (x700).

Fig. 20. Leaf impression of closed stoma from corn leaf exposed to 10^{-4}M solution of abscisic acid (ABA) for 1 hr, after being in a low CO_2 atmosphere for 48 hr (x945).

Table 11. Effects of exposure to 2 M potassium hydroxide (KOH), 10^{-4} M abscisic acid (ABA), and 10^{-4} M phenylmercuric acetate (PMA) on stomatal aperture size in corn leaves.

Treatment	Stomatal Aperture[1] (μm)	Range	Per cent Closed[2]
2 M KOH for 48 hr	4.2 a[3]	2.0-6.8	0
10^{-4} M ABA for 1 hr	0.4 b	0.0-1.6	60
10^{-4} M PMA for 1 hr	0.5 b	0.0-4.2	55

[1]Mean values for 400 measurements.

[2]Closed stoma = aperture of 1.0 μm or less.

[3]Mean stomatal apertures not followed by the same letter are significantly different ($P=0.01$) as determined by analysis of variance and by a LSD test.

Table 12. Per cent damaged leaf area in plants with open stomata is compared to per cent damaged leaf area in plants with closed stomata at time of inoculation with *Pseudomonas alboprecipitans*.

Stomatal Condition at time of Inoculation	Per cent Damaged Leaf Area
Open	34.3
Open	13.3
Open	26.3
Open	66.3
	$\bar{x}$ = 35.05 a[1]
Closed	24.0
Closed	7.3
Closed	41.3
Closed	20.7
	$\bar{x}$ = 20.7 a

[1]Mean per cent damage values not followed by the same letters are significantly different (P=0.05) as determined by analysis of variance.

DISCUSSION

The evidence is overwhelming that the whorl can be an important site for bacterial infection in corn. Others have described the whorl as an inoculation site in the study of bacterial diseases of grasses (21, 54, 62). In addition, natural epidemics of BLBSRC resulted in damaged areas of upper leaves without an effect on the leaves immediately below them (18). Examination of the infection process provided insight into why the lower leaves remained healthy. This study provided evidence that leaf areas in or not far removed from the whorl were the most favorable sites for infection by the BLBSRC bacterium.

Dew and guttation water that collected in the whorl provided a favorable habitat for *P. alboprecipitans*. Isolate PA 117 growing in whorl water increased to 10^8 cells/ml in 48 hr (Table 1) and respired at a rate equivalent to that when cultured in nutrient broth (Fig. 1). Therefore, whorl water was not inhibitory to *P. alboprecipitans*, and major growth factors (carbon and nitrogen sources) were detected in whorl water.

Ability of bacteria to move from the whorl into the leaf was established (Table 2). Because the marker (*S. marcescens*) was applied only to the whorl and over time found in upper portions of leaves, bacteria must have entered the leaf and moved upward with the flow of water. The variation which

existed was not unreasonable since bacterial movement appeared dependent upon plant-water relationships. Individual plants may vary in the distribution of water and water content even when subjected to exacting experimental conditions (50). Although variation occurred, bacterial movement up the leaf was dependent upon the exposure time. A trend existed, as the longer the corn plant was exposed to *S. marcescens*, the greater the probability that the red-pigmented bacterium would be isolated from upper leaf areas. Cytological evidence was not obtained, but it was speculated that the bacteria entered the leaf via stomata and moved upward in water-congested intercellular spaces. This speculation was based on the movement of fluorescent dye in water-congested intercellular spaces, when the dye was applied to the whorl.

Scanning electron micrographs of leaf surfaces from flooded whorl areas provided evidence that stomata were capable of opening even when submerged (Figs. 8-11). Autoradiographic evidence (Figs. 3-4) and SEM evidence (Figs. 12-17) proved *P. alboprecipitans* passed through stomata of whorl tissue. Furthermore, this was the most conclusive evidence that bacteria entered intercellular spaces via stomata. The usual evidence for stomatal ingress has been with histological studies of early colonized tissues (9, 51). Initial infections were traced back to sub-stomatal chambers; therefore, it was concluded that bacteria must have entered via stomata. This study provided two lines of evidence (autoradiography and SEM) which supported stomatal ingress by bacteria. The closure of stomata facilitated the observation of bacteria in the stoma, because

rod-shaped bacteria were found frequently in the basins formed by closed stomata. The only plausible explanation of how bacteria entered into sub-stomatal chambers is that they passed through open stomata. However, Panopoulos and Schroth (44) calculated that bacterial ingress in bean leaves reached equilibration in 1 hr, which meant that after 1 hr as many bacteria were moving out as were going into the leaves. This author observed bacteria in corn leaves only after a 160-320 min exposure. It was possible that bacteria entered earlier but were washed out by the techniques used for SEM. However, the few bacteria observed may have adhered to the tissue after an exposure of 160-320 min. Alternatively, ingress of *P. alboprecipitans* into corn may be slower than ingress of *P. phaseolicola* into bean.

Data from infectivity titration experiments (Table 4) also supported the hypothesis that the whorl was a favorable infection court. The level of inoculum (10^2 cells/ml) was low for a successful whorl inoculation. Spray inoculation required 10^6 cells/ml. No evidence existed which supported the idea that an increase in population of *P. alboprecipitans* was responsible for infection with low inoculum levels. Although the whorl is a favorable habitat for microbial survival and growth, the typical striped lesions appeared at the same time for all inoculum levels. If bacterial multiplication in the whorl was a prerequisite for infection at low inoculum levels, then lesion development should have been staggered over time. The staggered time periods would be due to the time required for bacteria to increase their population

to a level needed for infection. Apparently this was not the case, as all lesions appeared at the same time. However, disease severity was reduced in plants exposed to 10^5 cells/ml or less. Only one striped lesion appeared in plants exposed to low inoculum levels whereas blighted areas were extensive in plants exposed to higher inoculum levels (10^6 - 10^8 cells per ml).

The most significant data which implicated the whorl as an infection court were the results from local applications of PMA. Although results from field and greenhouse experiments were not identical, spray inoculations of the total plant resulted consistently in lesions only in leaves emerging from or in the whorl at time of inoculation. In no instances were lesions observed on leaves that were unfurled at time of inoculation. Moreover, a local application of bactericide (PMA) to the whorl temporarily protected plants in the field. Lesions were not observed above the whorl zones (leaf-zones 1 and 2) in either controls or treatments (Fig. 18). Plants treated with PMA had less disease and an altered distribution of lesion area compared to controls. However, the protection displayed by PMA was of short duration. Although data were not recorded, the emergence of two leaf-zones (20 cm) requires approximately 24-48 hr. Therefore, chemical protection of susceptible tissue would appear inefficient unless a bactericide had the property of redistribution. The redistribution of a bactericide so that it remained in the whorl possibly could control this disease. Otherwise, the rapid growth of corn would leave the infection court exposed,

and the nature of BLBSRC does not warrant the cost of several applications of a bactericide per week.

The role of the whorl as an infection court was not as clear in greenhouse experiments (Tables 5 and 6). Plants with a whorl treatment of PMA developed lesions above the whorl area (Table 7). This explained why disease severity was not affected as clearly by PMA in greenhouse tests when compared to field experiments. Areas immediately above the whorl zone could become infected under the conditions which existed in the greenhouse. Although bacterial migration up the leaf could explain lesions above the whorl, it does not afford an explanation for plants treated with PMA. The only plausible explanation is that bacteria entered the leaf above the whorl. Gitaitis (18) demonstrated that all corn leaves formed lesions if wounded or injected with a suspension of *P. alboprecipitans*. The author concluded that lesions usually did not form in older leaves, but that older leaves were susceptible. It was speculated that *P. alboprecipitans* could enter any leaf tissue under the proper conditions. Plants in the greenhouse were subjected to intense watering and high relative humidity prior to inoculation. These conditions were conducive for water-congestion. The importance of water-congestion for bacterial plant diseases has been documented (8, 30). Diachun et al. (11) demonstrated that not only bacteria entered water-congested leaf tissue, but india ink, toxic chemicals, and tobacco mosaic virus also entered the intercellular spaces of water-congested tissues. Corn and other plants often become water-congested due to high root-

pressure (29). Environmental conditions favorable for water uptake coupled with reduced plant transpiration are sufficient to induce water congestion. Therefore heavy rains are not required to predispose plants to bacterial infection. A pattern of water-congestion in corn can be one of translucent stripes emanating out of the whorl. Since all tissues are susceptible, the pattern of lesion development in or close to the whorl may be dependent upon criteria for ingress. Requirements for ingress (primarily water-congestion) may occur most often in juvenile tissues in or close to the whorl. There is precedent that epidemics are limited by factors that control ingress (22). In addition, predisposition to bacterial diseases has been examined by nitrogen-potassium imbalance (1) and differences in varietal responses to disease (26) have been explained by effects on water congestion. It is not inconceivable that ingress could be confined to the whorl due to that tissue becoming naturally water-congested more readily than older leaves. In any case, whether due to water-congestion or some other means, the leaf area in or immediately above the whorl was the most favorable infection court for *P. alboprecipitans*. The conditions that exist in the whorl are similar to those in the developing ear. Therefore, infection in the shank of the ear may be similar to events in the foliage. That is, infection may be dependent upon juvenile tissue and presence of free water.

Knowledge of the infection process could result in a wide range of applications. Many bacterial diseases of grasses (12, 13, 14, 31, 57), canna lily (6), fish-tail palm (33),

and bird-of-paradise (64) have etiologies similar to that of BLBSRC. Therefore, if a control measure can be developed for BLBSRC, in principle it could be effective against many bacterial diseases of plants with morphologies similar to corn.

Alternative control measures such as bio-control have possible application to this system. If an organism could colonize the whorl, disperse from plant to plant, and be antagonistic to *P. alboprecipitans*, it would be an effective bio-control agent. Although bio-control was not the main thrust of this study, it was examined briefly. Potentially, *E. herbicola* and *K. pneumoniae* were candidates to be antagonistic to *P. alboprecipitans* (10, 19). Results were not conclusive in terms of disease severity (Tables 5 and 6). However, *E. herbicola* did not affect lesion distribution (Table 7) nor did *E. herbicola* affect growth of *P. alboprecipitans* in nutrient broth (Table 8). There was no apparent antagonism when *E. herbicola* or *K. pneumoniae* were cultured with PA 73-31R. The use of a streptomycin-resistant mutant (PA 73-31R) was valuable in mixed culture studies. The population of PA 73-31R easily could have been underestimated or gone undetected if streptomycin had not been used to inhibit the saprophytes. Consequently, it would have been concluded erroneously that antagonism occurred in mixed culture. The negative results with the microorganisms in this study does not preclude bio-control as a possible control measure. All of the requirements for microbial growth occur in the whorl. It is recommended that antibiotic resistant mutants of *P. alboprecipitans* be used when screening for antagonists. Thus, interactions could be analyzed in terms of quantitative effects on the pathogen.

Stomata were identified as portals of entry for *P. alboprecipitans*. Therefore, it was logical that if the sites of ingress were closed then bacteria could not enter. However, stomatal manipulations failed to protect the plant (Table 12). Apparently a sufficient number of stomata (40-50 per cent) were open enough to allow bacteria access to the intercellular spaces. Results from SEM supported the conclusions that stomata in the whorl could be manipulated and that fully open stomata were not necessary for infection to occur. There were no apparent restraints of entry when stomata were partially open. Therefore, a stomatal inhibitor would have to be significantly more effective than 50-60 per cent closure in order to control this disease.

Another application which proved unfruitful was the use of the infection court to screen for resistant varieties. Neither movement of bacteria in leaves nor stomatal index was correlated with or related to known resistant varieties. The movement of markers (dye or microorganisms) in leaves of resistant and susceptible varieties may still have application if exacting conditions are worked out. However, there appears to be too much variation in disease response to use it as an effective screening procedure for commercial operations.

While not attempted here, the most effective screening procedure may be with infectivity titration. Ercolani (15) proposed infectivity titration as a procedure to assess disease resistance. This study identified the infection court and portals of entry which would be required information for infectivity titration. In addition, it was found that per

cent leaf damage in varieties grown in pots in the greenhouse (Table 10) matched the reported (54) response in the field. Therefore, whorl inoculation with infectivity titration may be the most effective means of screening for resistance to *Pseudomonas alboprecipitans*.

In conclusion, a number of types of evidence incriminated the whorl as an infection court for *Pseudomonas alboprecipitans*. Most significant was the protection by whorl applications of PMA and the lack of infection in lower unfurled leaves. Furthermore, conclusive evidence of stomatal entry was obtained by two techniques. Both SEM and autoradiography are valuable tools which should be used in future studies of bacterial plant diseases. Although there were no significant control procedures developed from the initial attempts in this study, the facts reported here may contribute to the development of controls in the future.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James O. Strandberg, Chairman
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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